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Clusterin is a multifunctional disulphide linked protein that is induced during regression of hormone dependent tissues. Of particular interest to us is the observation that clusterin expression is confined to surviving cells following induction of cell death, suggesting that it may be involved in cell survival rather than cell death. Clusterin expression has also been correlated with tumor grade and resistance to cytotoxic compounds such as TNF-alpha in prostate cancer.

In our studies we have focused on determining whether clusterin plays a causative role in the progression of human breast carcinoma by promoting cell survival, increasing cell motility and resistance to cytotoxic drugs. Our studies have utilized an ER-alpha positive non-invasive MCF-7 cell line, an MCF-7 cell line genetically engineered to overexpress clusterin (MCF-7CLU) and an ER-alpha negative invasive SUM-159PT cell line. Our major finding to date are that SUM-159PT and MCF-7CLU cells secrete 5-10 times more clusterin than MCF-7 cells. Both SUM-159PT and MCF-7CLU cells display resistance to TNF-alpha in comparison to the highly sensitive MCF-7 cells. Furthermore, our *in vitro* invasion assays demonstrate a dramatic increase (10 fold) in the invasive potential of the MCF-7CLU cells as compared to the parental non-invasive MCF-7 cells. Our data clearly demonstrate a role for clusterin in breast tumor promotion and resistance to possible thereapeutic compounds. *In vivo* studies currently underway are focusing on tumor growth and progression in the MCF-7 vs MCF-7CLU cell lines, followed by measuring sensitivity to the most commonly used clinical antiestrogen tamoxifen.

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## INTRODUCTION

Metastatic spread of cancer continues to be the greatest barrier to cancer cure. It is ultimately the metastatic process that causes mortality of breast cancer since uncontrolled growth of malignant cells in other tissues continues at the expense of the normal function of that tissue. Understanding the molecular mechanisms of metastasis is crucial for the design and effective use of novel therapeutic strategies to combat cancer.

Clusterin is a multifunctional disulphide linked glycoprotein that is induced during regression of hormone dependent tissues (Ahuja *et al.*, 1994; Buttyan *et al.*, 1989). Of particular interest to us is the observation that clusterin expression is confined to surviving cells following induction of cell death, suggesting that it may be involved in cell survival rather than death. Clusterin expression correlates with tumor grade in prostate cancer and in breast cancer cells where clusterin expression or protection has been less extensively studied, clusterin expression is associated with large tumor size, lack of estrogen and progesterone receptor status and with the progression from primary carcinoma to metastatic carcinoma in lymph nodes (Steinberg *et al.*, 1997; Redondo *et al.*, 2000). The increased clusterin expression in breast carcinomas correlates inversely with the apoptotic index, which indicates that clusterin gene expression is not a prerequisite to cellular death by apoptosis. This data indicates clusterin expression may have a role in tumorigenesis and progression of human breast carcinoma. However to date, there is little other data available on the effects of clusterin expression and secretion on human breast cancer growth, invasion and sensitivity to cytotoxic drugs *in vitro* or *in vivo*. Sensitivity to TNF-$\alpha$ in PC3 and LNCaP prostatic cancer cells has been shown to be regulated by extracellular levels of clusterin (Sintich *et al.*, 1999). It has also been shown that overexpression of the clusterin gene in human androgen-dependent LNCaP prostate cancer cells by stable transfection rendered them highly resistant to androgen ablation *in vivo*, and *in vivo* administration of anti-sense oligonucleotides and either paclitaxel or mitoxantrone significantly decreased the primary tumor volume by approximately 70% respectively compared to mismatch control oligonucleotides plus either paclitaxel or mitoxantrone (Miyake *et al.*, 2000a; Miyake *et al.*, 2000b; Miyake *et al.*, 2000c). These findings strongly suggest that clusterin overexpression confers resistance to cytotoxic chemotherapy in prostate cancer cells.

The scope of these studies will examine whether clusterin plays a role in tumorigenesis and progression of human breast carcinomas by promoting cell survival, increasing cell motility and increasing resistance to cytotoxic drugs. Inhibition of the secretion of clusterin using anti-sense technology or siRNA in breast cancer cells may therefore enhance the sensitivity of breast cancer cells to therapeutic agents, induce more rapid tumor regression, and possibly inhibit the metastatic spread of breast cancer. We anticipate that these studies will confirm that clusterin overexpression confers resistance to cytotoxic drugs in breast cancer cells and will illustrate the potential utility of combined treatments of blocking clusterin expression with other therapeutic agents for patients with both hormone-dependent and hormone refractory breast cancer.

## ANNUAL SUMMARY-BODY

Based on our literature searches and preliminary data carried out for the grant proposal we suggest that clusterin expression may play a role in tumorigenesis and progression of human breast carcinomas. The aim of this research proposed here is to determine whether clusterin expression in breast cancer cells confers a survival advantage and helps to accelerate the progression to a more, invasive drug-resistant phenotype.

Previously in our grant proposal we were utilizing two breast cancer cell lines from different stages of breast cancer progression and with different sensitivity to cytotoxic drugs. MCF-7 cells are an ER-alpha-positive, non-invasive breast cancer cell line that form localized tumors after orthotopic injection into the mammary fat pads of nude mice in the presence of estradiol. SUM-159PT cells on the other hand are ER-alpha negative, tumorigenic and metastatic in ovariectomized nude mice in the absence of estradiol. We demonstrated previously that MCF-7 and SUM-159PT cells exhibit varying sensitivities to cytotoxic compounds, for example SUM-159PT cells were resistant to the cytotoxic drug TNF-$\alpha$ whereas the ER-positive MCF-7 cells are highly sensitive. Of particular interest to us and pertaining specifically to specific aim 1 of this proposal is that clusterin levels from concentrated media from SUM-159PT cells treated with TNF-$\alpha$ for 48h were significantly increased when compared to EtOH treated control SUM-159PT cells. Although preliminary, this data indicated a possible protective effect of clusterin on the SUM-159PT cells following TNF-$\alpha$ treatment.

My current research has expanded on the above findings. Our lab expanded our study on the role of clusterin in breast cancer by creating an MCF-7 cell line genetically engineered to overexpress clusterin to compare solely the effects of clusterin overexpression on the MCF-7 cell line, whilst also using the SUM-159PT cells as a model for invasive, metastatic and cytotoxic drug resistant breast cancer.

In our laboratory we have a well designed protocol for overxpression of genes using Gateway technology. Using Invitrogen's Gateway Technology, which allows for the transfer of DNA among different vectors based on lambda-phage recombination, the clusterin gene was inserted into a donor vector (pDONOR 201) and then subcloned into a mammalian expression vector which was then stably transfected into the MCF-7 cell line using Lipofectamine 2000 transfection reagent.

Chararcterization of this MCF-7CLU cell line displayed several important factors. Firstly we examined the basic morphology of these cells using phase microscopy. As demonstrated in Figure 1, MCF-7CLU cells exhibit more cell to cell contacts and form a tighter knit monolayer (Panels D, E and F) as compared to the islands of cells exhibited by the parental MCF-7 cells (A, B and C).

We next examined the growth rates of MCF-7 cells compared to MCF-7CLU and SUM-159PT cells and their sensitivity to the cytotoxic compound TNF-$\alpha$. Using crystal violet assays we demonstrated no difference in the growth rates between MCF-7CLU and MCF-7 cells. SUM-159PT cells proliferate at a faster rate than either of the MCF-7 cell

lines (Figure 2). Treatment of MCF-7 cells with TNF-α for 48 h dramatically decreases cell number to 30% of EtOH treated cells. As previously demonstrated in our grant proposal SUM-159PT cells are resistant to the growth inhibitory effects of TNF-α. Of particular interest is the observation that MCF-7CLU cells are also resistant to the growth inhibitory effects of TNF-α (Figure 2). This critical piece of data confirms that clusterin overexpression confers resistance to cytotoxic drugs in breast cancer cells. These data are consistent with previous work carried out in prostate cancer cells demonstrating that sensitivity to TNF-α in PC3 and LNCaP prostatic cancer cells is regulated by extracellular levels of clusterin (Steinberg *et al.*, 1997; Sintich *et al.*, 1999).

Preliminary data from our lab has also demonstrated that MCF-7CLU exhibit partial resistance to the clinically used anti-estrogen Tamoxifen (data not shown). These studies are currently been reproduced.

Expanding on this data we examined intra- and extra-cellular levels of clusterin in MCF-7, MCF-7CLU and SUM-159PT cells. As demonstrated in Figure 3A, MCF-7CLU cells manufacture intra-cellular clusterin at a 5-10 fold higher rate than that observed in MCF-7 cells. In contrast, intracellular levels of clusterin in SUM-159PT cells are considerably lower than that observed in either the MCF-7 or MCF-7CLU cell line. Treatment with TNF-α induces a 2-3 fold increase in MCF-7 intra-cellular clusterin levels. In contrast no increase in intra-cellular levels of clusterin is observed in SUM-159PT cells. Examination of the levels of intra-cellular clusterin in TNF-α treated MCF-7CLU are currently been carried out.

Examination of extra-cellular levels of clusterin demonstrated that MCF-7CLU clusterin protein expression is approximately 10-15 fold higher than that observed in the parental MCF-7 cells (Fig 3B). SUM-159PT also expressed dramatically higher levels (5-8 fold) of extra-cellular clusterin as compared to the parental MCF-7 cell line, demonstating that SUM-159PT cells process clusterin differently than the MCF-7 cells as the intra-cellular levels of clusterin in SUM-159PT cells were lower than that observed in MCF-7 cells however they secrete much higher levels than the MCF-7 cells. Treatment of MCF-7 cells with TNF-α causes a 3-5 fold increase in extra-cellular clusterin levels which is consistent with that observed in the cytosolic (intra-celluar) fraction. Examination of the levels of intra-cellular clusterin in TNF-α treated MCF-7CLU are currently been carried out. Treatment of SUM-159PT cells with TNF-α however did not upregulate extracellular clusterin levels as compared to untreated SUM-159PT cells however the levels of clusterin are still significantly higher than that observed in TNF-α treated MCF-7 cells indicating that SUM-159PT cells constitutively secrete high levels of clusterin extracellularly. This piece of data is not in accordance with our previous preliminary data that demonstrated an increase in extra-cellular clusterin levels upon TNF-α treatment however we are currently using a new mouse monoclonal antibody (RDI-CLUSTabm-41D from research diagnostic labs and this data has been reproduced 3 times.

Our data clearly demonstrate therefore that high extracellular levels of clusterin confers resistance to the cytotoxic drug TNF-$\alpha$ and is consistent with data from prostate cancer model systems that have shown that overexpression of the clusterin gene in human androgen-dependent LNCaP prostate cancer cell lined rendered them highly resistant to androgen ablation *in vivo* (Miyake *et al.*, 2000b; Mikaye *et al.*, 2000c).

Our next aim was to examine if the increased expression of clusterin in MCF-7CLU and SUM-159PT cells confers a survival advantage and helps accelerate the progression to more invasive metastatic phenotype. As demonstrated in Figure 4, in a modified Boyden chamber invasion assay, MCF-7 cells invade minimally, in contrast to the highly invasive SUM-159PT cells. Overexpression of clusterin in MCF-7 cells caused a 10-fold increase in the level of invasion as compared to parental MCF-7cells.

Taken together our data are the first to clearly demonstate in breast cancer cells that clusterin expression confers a survival advantage and helps to accelerate the progression to a more invasive drug-resistant phenotype. Our data are consistent with the single report in breast tumor specimens that clusterin expression is associated with large tumor size, lack of estrogen and progesterone receptor status and with the progression from primary carcinoma to metastatic carcinoma in lymph nodes (Redondo *et al.*, 2000).

Expanding on our *in vitro* data, we have designed and are currently carrying out an *in vivo* study to determine if the overexpression of clusterin in MCF-7 cells and SUM-159PT cells affects tumor take rate, tumor doubling time and metastatic potential in each of the cell lines. We are also currently testing whether clusterin expression affects sensitivity to the most commonly used clinical antiestrogen, tamoxifen in MCF-7, MCF-7CLU and SUM-159PT cells. As shown in Figure 5 we have inoculated Ncr nude mice in the inguinal mammary fat pad with fluorescently tagged MCF-7RFP cells, MCF-7CLURFP and SUM-159PTGFP cells to allow us to track metastatic spread.

**Study Design:**

| | |
|---|---|
| **No. of animals to be ordered:** | 100 |
| **Strain:** | Female NCRNU-M (Homozygotes) |
| **Surgeries prior to arrival:** | All animals will be ovariectomized by the supplier and implanted with 1.7mg (90 days) 17-β estradiol pellets (Innovative Research of America). Extra 1.7mg 17-β estradiol pellets will be ordered for the studies involving tumor growth and metastatic progression **(A).** |
| **Duration of studies:** | For studies investigating tumor growth, progression, metastatic progression **(A-see below)**, these studies will continue for a maximum of 12 weeks from the time of tumor cell inoculation. For studies involved with investigating the effects of tamoxifen on tumor growth (**B-see below**), these studies will continue for |

a maximum of 9 weeks from the time of tumor cell inoculation.

**A.** A total of 33 animals will be used to measure tumor take rate, tumor doubling times, and metastatic progression in three groups of nude mice inoculated with MCF-7RFP, MCF-7RFP$^{CLU}$ or SUM-159PTGFP cells. Nude mice will be inoculated in the inguinal mammary fat pad under anesthetic (rodent cocktail (0.2cc per mouse)) using a 1cc syringe and 26 gauge needle with MCF-7RFP, MCF-7RFP$^{CLU}$ or SUM-159PTGFP cells suspended in Matrigel. Tumor volumes will be measured weekly by caliper measurements. After approximately 12 weeks of tumor growth, mice will be euthanized using $CO_2$, followed by cervical dislocation. Tumors, lungs, liver and lymph nodes will be removed and either be fixed in formalin or frozen using liquid nitrogen for metastases detection and further histological and biochemical analysis.

**B.** A total of 66 animals will be used to measure the effects of tamoxifen on tumor progression. Nude mice will be inoculated in the inguinal mammary fat pad under anesthetic (rodent cocktail (0.2cc per mouse)) using a 1cc syringe and 26 gauge needle with MCF-7RFP, MCF-7RFP$^{CLU}$ or SUM-159PT cells suspended in Matrigel. Tumor volumes will be recorded weekly by caliper measurements. When tumors reach approximately 200mm$^3$ (3-4 weeks, mice from each group will be assigned to control or tamoxifen treatment groups. Anesthetized mice will be implanted intraperitoneally with placebo or 15 mg tamoxifen (60 days) pellets. Tumor volumes and mouse weights will be monitored weekly. Tumors, lungs, liver and lymph nodes will be removed and fixed in formalin or frozen using liquid nitrogen for metastases detection and further histological and biochemical analysis.

In summary our work to date has demonstrated that clusterin expression plays a causative role in the progression of human breast cancer. We have clearly demonstrated clusterin expression is correlated with increased cell survival, increased invasiveness and increased resistance to cytotoxic drugs. We hope that our current *in vivo* studies will be consistent with our in vitro work.

## STATEMENT OF WORK

**Task 1: To quantitate the relative levels of clusterin secretion in conditioned media of either untreated (control) or treated (TNF-α, Vitamin D) SUM-159PT and MCF-7 cells.**

My studies so far have allowed me to measure using western blot analysis the relative secretion of clusterin in conditioned media of not only control and TNF-α treated MCF-7 and SUM-159PT cells but also from the newly genetically engineered MCF-7 cell line that overexpresses clusterin (see above- Figure 2).

Our future studies plan to repeat the above studies using not only vitamin D treated cells but also tamoxifen treated cells as tamoxifen is the most commonly used clinical endocrine therapy for women with breast cancer and so to examine the role of clusterin in relation to sensitivity/resistance to tamoxifen is of great clinical importance.

**Task 2: To determine the effects of AS clusterin on growth, invasion and sensitivity to cytotoxic compounds in SUM-159PT and MCF-7 tumors.**
I am currently stably transfecting the SUM-159PTGFP and MCF-7RFP cells with AS clusterin under the control of the Tet/on system and once this is completed Task 2b-e will be completed. Our lab has however created an MCF-7 cell line that has been engineered to overexpress clusterin and our results have demonstrated that overexpression of clusterin leads to increased survival, invasion and resistance to cytotoxic compounds.

Task 3 and 4 will be completed once task 2 is done.

## KEY RESEARCH ACCOMPLISHMENTS

- Creation by our laboratory of an MCF-7 cell line genetically engineered to overexpress clusterin, designated MCF-7CLU.

- Using cell proliferation assays, we have demonstrated that MCF-7 cells are highly sensitive to the anti-proliferative effects of the cytotoxic compound TNF-α. The invasive SUM-159PT cell line are resistant to the effects of TNF-α. Similarly MCF-7CLU have acquired resistance to the cytotoxic effects of TNF-α indicating that the overxpression of clusterin is directly related to cell survival and resistance to cytotoxic drugs.

- Measurement of intra-cellular and extra-cellular levels of clusterin has demonstrated that MCF-7 cells express and secrete relatively low levels of clusterin but are upregulated in response to treatment with TNF-α. SUM-159PT express low intra-cellular levels of clusterin in both control and TNF-α treated cells. Extra-cellular levels of clusterin expression in SUM-159PT cells are also not altered between control and TNF-α treated cells however the overall level of clusterin extracellular expression is 5-10 fold higher than that observed in MCF-7 cells. The constitutively high level of clusterin expression in SUM-159PT cells must therefore confer continual resistance to cytotoxic compounds such as TNF-α. MCF-7CLU cells manufacture and secrete extremely high levels of clusterin, up to 10-fold higher intra-cellularly and up to 15 fold higher extracellularly when compared to the parental MCF-7 cell line demonstrating that the increase in extracellular clusterin expression is correlated with resistance to cytotoxic compounds.

- Using invasion assays, we have demonstrated that MCF-7CLU are up to 10-fold more invasive than MCF-7 cells indicating a direct role for clusterin in tumor progression and invasive potential.

## REPORTABLE OUTCOMES

- **Development of a new cell line:** In our laboratory we have a well designed protocol for overxpression of genes using Gateway technology. Using Invitrogen's Gateway Technology, which allows for the transfer of DNA among different vectors based on lambda-phage recombination, the clusterin gene was inserted into a donor vector (pDONOR 201) and then subcloned into a mammalian expression vector which was then stably transfected into the MCF-7 cell line using Lipofectamine 2000 transfection reagent. This new cell line is designated **MCF-7CLU**.

## CONCLUSIONS

The overall aim of our studies was to investigate whether clusterin expression plays a causative role in the progression of human breast carcinoma.

Our data are the first to show in breast cancer cells that high extracellular clusterin expression plays a role in tumorigenesis and progression of human breast cancer carcinomas by promoting cell survival, increasing cell motility and invasion and resistance to cytotoxic drugs.

Our data therefore would illustrate the potential utility of combined treatment of anti-sense (AS) clusterin and other therapeutic agents for patients with either hormone-dependent or hormone refractory breast cancer. For patients with hormone-dependent breast cancer, we would predict lower levels of therapeutic agents in conjunction with AS clusterin would yield a similar result as higher levels of drug alone, thereby minimalizing some of the potentially unfavorable side effects of many currently used therapeutic agents. In the case of hormone-refractory breast cancers we would predict an enhanced sensitivity to cytotoxic drugs and an inhibition of metastatic spread of breast cancer cells. In addition since clusterin is a secreted protein and required for cell survival, support for this hypothesis would suggest that clusterin is an excellent target for immumotherapy.

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## APPENDIX 1- FIGURES

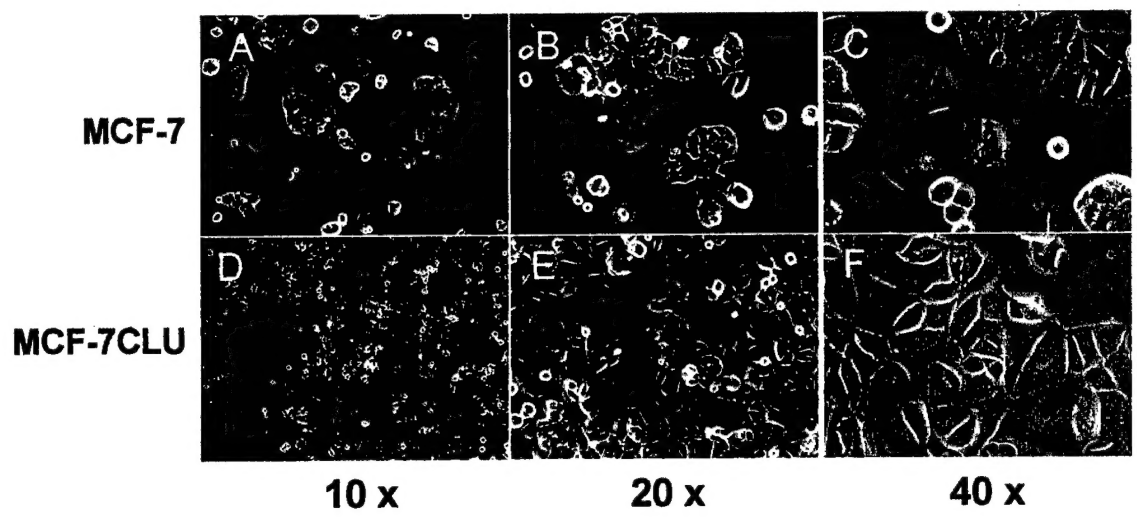


**Figure 1:** Phase Microscopy of MCF-7 and MCF-7CLU cells. MCF-7 and MCF-7 cells were plated on Lab-Tek cell culture inserts at 20,000 cells/ml and allowed to grow for 72 h. Cells were fixed with 4% formalin and photographed using phase microscopy at 10 x, 20 x and 40 x magnification.

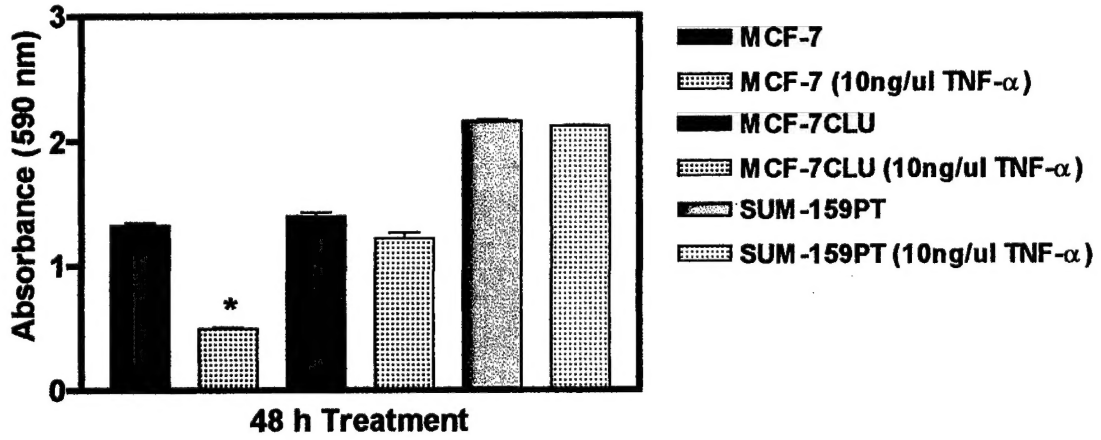


**Figure 2:** Effects of 10 ng/μl TNF-α on MCF-7, MCF-7CLU and SUM-159PT cell growth. Cells were plated at 20,000 cells/ml and two days after plating cells were treated with either ethanol vehicle or 10 ng/μl TNF-α for 48 h. Total cell number was determined by crystal violet staining. Data represent mean ±SEM of four values per time point. *, $P<0.001$; ethanol control vs TNF-α treated.

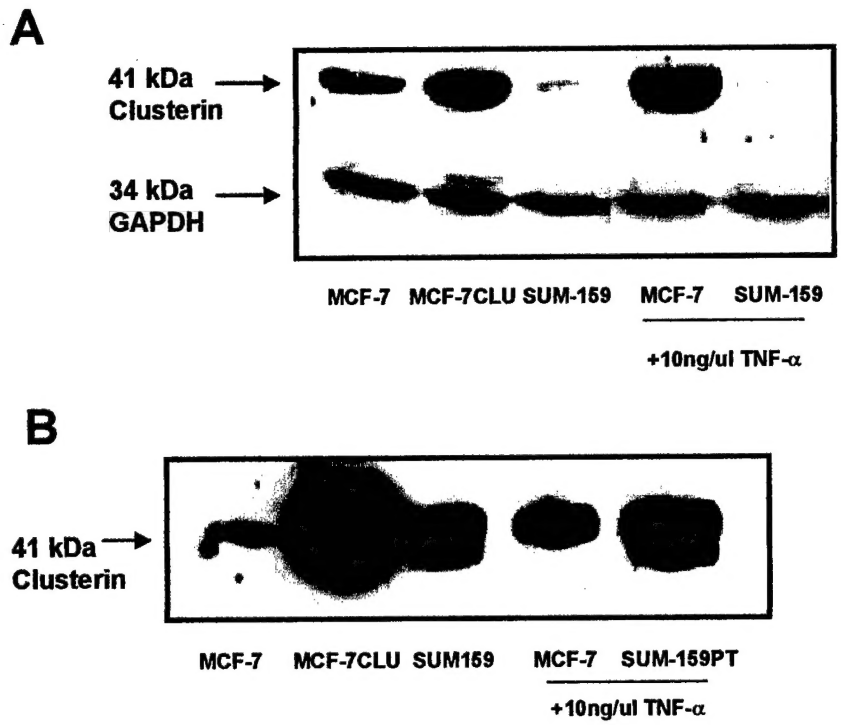


**Figure 3: Western Blot Analysis of Clusterin in Cell Lysates (A) and Conditioned Media (B).** MCF-7, MCF-7CLU and SUM-159PT cells were ethanol control treated or MCF-7 and SUM-159PT were treated with 10 ng/μl TNF-α for 24 h and separated on a 12.5% SDS-PAGE gel and immunoblotted with a mouse monoclonal antibody directed against clusterin (A and B) and GAPDH (A – to confirm equal loading).

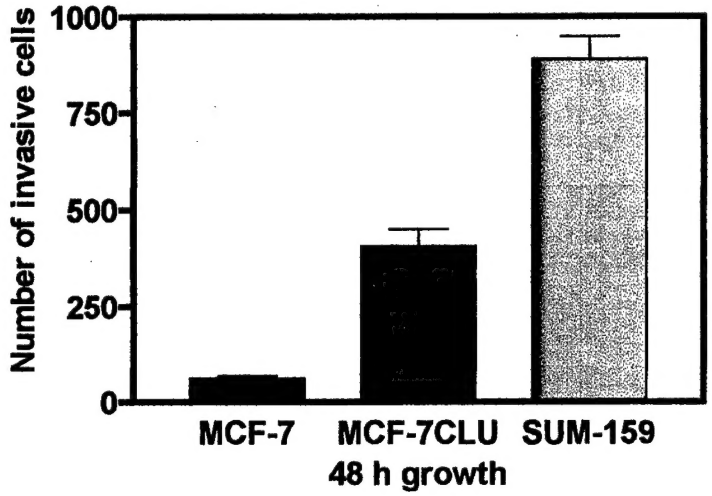


**Figure 4: Invasive Potential of MCF-7, MCF-7CLU and SUM-159PT cells.** Cells were analyzed in the Boyden chamber invasion assay. 50,000 cells/ml were plated in each insert and cells were allowed to grow for 48 h. Cells on the underside of the insert were fixed with 1% gluteraldehyde, stained using crystal violet, allowed to dry and cells counted using a light microscope. Data represent mean ±SEM of four values per time point.

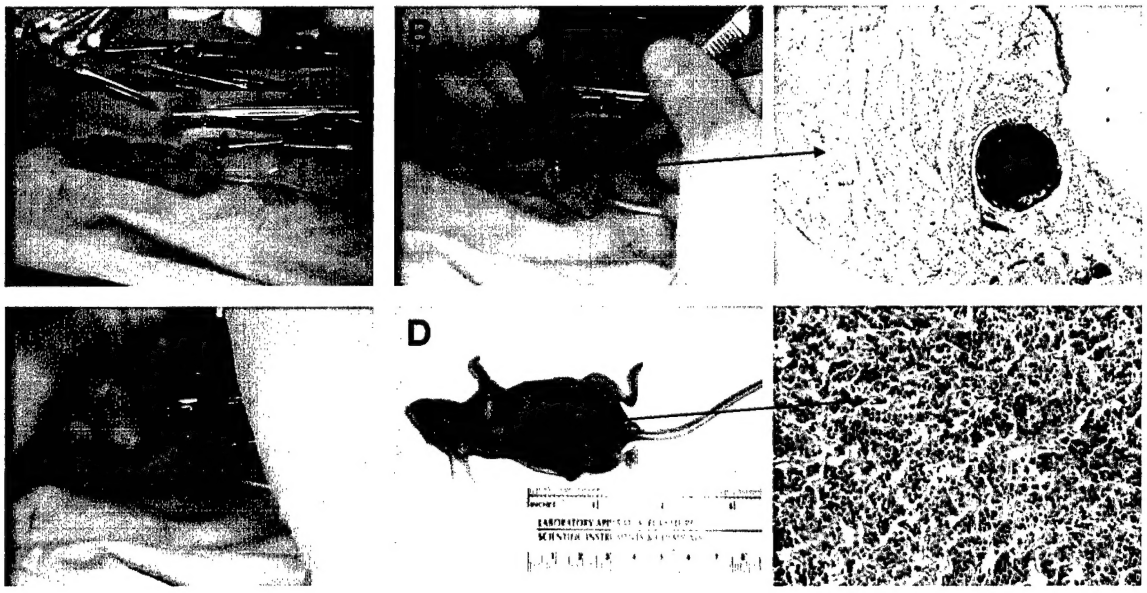


**Figure 5: Inoculation of breast cancer cells into the inguinal mammary fat pad of nude mice.** MCF-7, MCF-7CLU and SUM-159PT cells suspended in matrigel were injected into mammary fat pad using a 26 gauge needle and a 1 cc syringe.